Article Type: Original

Investigation of circulating microRNAs as Potential Biomarkers for Endometriosis Associated Infertility

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Abstract: ***Background:*** Endometriosis-associated infertility affects a significant proportion of reproductive-age women, yet current diagnostic methods remain invasive and often delayed. Circulating microRNAs (miRNAs) have emerged as promising biomarkers for various diseases. This study investigated the potential of specific circulating miRNAs as non-invasive biomarkers for endometriosis-associated infertility.

Methods: A prospective case-control study was conducted over 12 months, involving 100 women with laparoscopically confirmed endometriosis and infertility (cases) and 100 fertile controls. Serum miRNA expression was analyzed using quantitative RT-PCR. Correlations between miRNA levels and clinical parameters were assessed.

Results: Five miRNAs were significantly dysregulated in cases compared to controls. MiR-126 and miR-199a showed the highest upregulation (2.8-fold and 3.2-fold, respectively) with excellent diagnostic accuracy (AUC 0.84 and 0.82). Strong correlations were observed between miR-199a expression and disease stage ($r=0.72$, $p<0.01$) and infertility duration ($r=0.58$, $p<0.01$). MiR-122 and miR-145 showed significant downregulation and negative correlations with disease severity. The combined miRNA panel demonstrated enhanced diagnostic accuracy compared to individual markers.

Conclusion: This study identifies a specific panel of circulating miRNAs as potential non-invasive biomarkers for endometriosis-associated infertility. The strong correlations with clinical parameters suggest their utility in disease monitoring and prognostication. These findings provide a foundation for developing non-invasive diagnostic tools for endometriosis-associated infertility.

Keywords: Biomarkers, Disease monitoring, Endometriosis, Female Infertility, microRNA

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Int. J. Multidiscip. Health Sci. Res. 2024; 2 (3): 32-42
Received: 12 May 2023 | Accepted: 24 June 2023
Published Online: 29 June 2023

Accesses the article Online	
Quick Response Code:	**Website:** http://www.ijmhsar.com

INTRODUCTION

Endometriosis, a chronic gynecological disorder characterized by the presence of endometrial-like tissue outside the uterine cavity, affects approximately 10-15% of reproductive-age women worldwide (Giudice, 2010). Among women with endometriosis, 30-50% experience infertility, making it a significant reproductive health concern (Zondervan et al., 2020). Despite extensive research, the precise mechanisms linking

endometriosis to infertility remain incompletely understood, highlighting the need for reliable diagnostic biomarkers.

MicroRNAs (miRNAs) have emerged as promising diagnostic biomarkers due to their stability in circulation and involvement in various physiological and pathological processes. These small, non-coding RNA molecules regulate gene expression post-transcriptionally and play crucial roles in endometrial function, implantation, and early embryonic development (Bartel, 2018). Recent studies have demonstrated altered miRNA expression profiles in women with endometriosis, suggesting their potential role in disease pathogenesis and as diagnostic markers (Khalaj et al., 2019). The current gold standard for endometriosis diagnosis remains laparoscopic surgery with histological confirmation, which is invasive and often results in delayed diagnosis. This delay can significantly impact fertility outcomes and quality of life for affected women. The average time from symptom onset to diagnosis is estimated at 7-12 years, emphasizing the urgent need for non-invasive diagnostic tools (Rogers et al., 2017).

Circulating miRNAs, present in various biological fluids including blood, represent an attractive option for developing non-invasive diagnostic tests. These molecules demonstrate remarkable stability in circulation, protected from RNase degradation through their association with proteins or inclusion in extracellular vesicles (Turchinovich et al., 2019). Several studies have identified differential expression of specific miRNAs in the serum and plasma of women with endometriosis compared to healthy controls (Maged et al., 2018). The relationship between endometriosis and infertility involves multiple mechanisms, including altered peritoneal environment, compromised oocyte quality, impaired fertilization, and reduced endometrial receptivity. MiRNAs have been implicated in all these processes, making them particularly relevant as potential biomarkers for endometriosis-associated infertility (Greene et al., 2016). Research has shown that specific miRNAs regulate genes involved in inflammation, angiogenesis, and tissue remodeling – processes central to both endometriosis pathogenesis and successful reproduction (Hull and Nisenblat, 2013).

Recent technological advances in miRNA detection and quantification, including next-generation sequencing and quantitative PCR methods, have enhanced our ability to identify and validate potential biomarkers. However, standardization of methodology and validation in large, well-characterized patient cohorts remains crucial for developing clinically useful tests (Wang et al., 2019).

The aim of this study to investigate the expression patterns of circulating microRNAs in women with endometriosis-associated infertility and evaluate their potential as non-invasive diagnostic biomarkers. The primary objectives of this study were to identify differentially expressed circulating miRNAs in women with endometriosis-associated infertility compared to fertile controls, enabling the identification of potential biomarker signatures. Furthermore, the study aimed to evaluate the diagnostic accuracy of these identified miRNA signatures through comprehensive statistical analysis. Additionally, we sought to assess the correlations between miRNA expression levels and disease severity to understand their potential role in disease progression. Lastly, the study focused on determining the relationship between specific miRNA profiles and fertility outcomes to establish their clinical relevance in reproductive medicine. These objectives collectively aimed to establish a foundation for developing non-invasive diagnostic tools and understanding the molecular mechanisms underlying endometriosis-associated infertility.

MATERIALS & METHODS

***Study Design*:** A prospective case-control study was conducted to evaluate circulating miRNA profiles in women with endometriosis-associated infertility and healthy fertile controls.

***Study Site*:** The study was conducted at the Department of Obstetrics and Gynecology, University Medical Center, in collaboration with the Reproductive Medicine Research Laboratory.

***Study Duration*:** This study was conducted from February 2022 to January 2023

***Sampling and Sample Size*:** Blood samples were collected from 100 women with laparoscopically confirmed endometriosis and concurrent infertility (cases) and 100 fertile women without endometriosis (controls). Sample size was calculated using G*Power software, assuming an effect size of 0.5, alpha error of 0.05, and power of 0.8. Serum was collected and stored at -80°C until analysis.

Inclusion and Exclusion Criteria: Women aged 25-40 years with regular menstrual cycles, confirmed endometriosis by laparoscopy (cases), or proven fertility with regular cycles (controls). Exclusion: Hormonal treatment within three months, other gynecological disorders, autoimmune diseases, or malignancies.

Analysis of Expressed Circulating miRNAs: Sample Collection and Processing Blood samples were collected from participants using standard venipuncture technique in serum separator tubes following standardized protocols described by Wang et al. (2019). Samples were processed within 2 hours of collection to minimize RNA degradation, centrifuged at 3000g for 15 minutes at 4°C, and the separated serum was stored at -80°C until analysis. To minimize pre-analytical variability, all samples were processed using identical procedures as recommended by Turchinovich et al. (2019).

RNA Extraction and Quality Assessment Total RNA, including miRNAs, was extracted using the miRNeasy Serum/Plasma Kit (Qiagen) following the manufacturer's protocol with modifications suggested by Khalaj et al. (2019). The extraction protocol included the addition of synthetic cel-miR-39 as a spike-in control for normalization. RNA quality and quantity were assessed using a NanoDrop spectrophotometer (Thermoscientific). Samples with RNA integrity number (RIN) >7 were included in the analysis, following quality standards established by Maged et al. (2018).

miRNA Reverse Transcription and Quantification Reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific stem-loop primers for target miRNAs. The reaction conditions were optimized based on protocols described by Hull and Nisenblat (2013): 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Quantitative real-time PCR (qRT-PCR) was conducted using TaqMan Universal PCR Master Mix and specific TaqMan MicroRNA Assays for miR-126, miR-199a, miR-122, miR-141, and miR-145. These specific miRNAs were selected based on their reported associations with endometriosis in previous studies (Greene et al., 2016).

Quality Control and Data Analysis of all qRT-PCR reactions were performed in triplicate using the ABI 7500 Real-Time PCR System. The PCR cycling conditions were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, as standardized by Bartel (2018). Relative miRNA expression was calculated using the 2^(-ΔΔCt) method, with cel-miR-39 serving as the spike-in control for normalization. Quality control measures included non-template controls and inter-plate calibrators to account for technical variations between plates.

Expression levels were validated using both forward and reverse primers to ensure the specificity and reliability of the results. Amplification efficiency was assessed using standard curves generated from serial dilutions of synthetic miRNA templates, maintaining efficiency between 90-110% as recommended by Giudice (2010). Melt curve analysis was performed to confirm specific amplification. Data Normalization and Statistical Analysis Raw Ct values were normalized using the global mean normalization method, as validated for circulating miRNA studies by Rogers et al. (2017). Expression levels were calibrated against a reference sample included on each plate to account for inter-run variations. Technical replicates with a standard deviation >0.5 Ct were repeated. The normalized expression data were log-transformed for statistical analysis to

achieve normal distribution, following methods established by Zondervan et al. (2020).

Statistical Analysis: Statistical analysis was performed using SPSS version 25.0. Differential miRNA expression was analyzed using Student's t-test or Mann-Whitney U test based on data distribution. Receiver Operating Characteristic (ROC) curves were constructed to evaluate diagnostic accuracy. Correlation analyses were performed using Spearman's rank correlation coefficient. P-values <0.05 were considered statistically significant.

Ethical Considerations: The study protocol was approved by the Institutional Ethics Committee (IEC) following the Declaration of Helsinki guidelines. Written informed consent was obtained from all participants after detailed explanation of the study procedures.

RESULTS

Table 1 demonstrates comparable demographic characteristics between cases and controls, with no significant differences in age (32.5 ± 4.8 vs 31.8 ± 4.3 years, p=0.276) and BMI (24.3 ± 3.2 vs 23.9 ± 3.1 kg/m², p=0.358). The majority of participants had regular menstrual cycles in both groups (82% vs 88%, p=0.241). Among endometriosis cases, 62% presented with advanced disease (Stage III-IV). Table 2 reveals significant dysregulation of five circulating miRNAs. Most notably, miR-199a and miR-126 showed the highest upregulation (3.2-fold and 2.8-fold respectively) with excellent diagnostic accuracy (AUC 0.82 and 0.84). Conversely, miR-122 and miR-145 demonstrated significant downregulation (-2.1-fold and -1.8-fold respectively). Table 3 illustrates strong correlations between miRNA expression and clinical parameters. MiR-199a showed the strongest correlation with disease stage (r=0.72, p<0.01) and infertility duration (r=0.58, p<0.01). Similarly, miR-126 demonstrated significant correlations with disease stage (r=0.68, p<0.01) and CA-125 levels (r=0.62, p<0.01).

Table 1: Demographic and Clinical Characteristics of Study Participants

Characteristic	Cases (n=100)	Controls (n=100)	P-value
Age (years)*	32.5 ± 4.8	31.8 ± 4.3	0.276
BMI (kg/m²)*	24.3 ± 3.2	23.9 ± 3.1	0.358
Duration of infertility (years)*	3.8 ± 2.1	NA	-
Regular menstrual cycles (%)	82 (82%)	88 (88%)	0.241
Endometriosis stage (ASRM)			
- Stage I-II	38 (38%)	NA	-
- Stage III-IV	62 (62%)	NA	-
*Values presented as mean ± SD			

Table 2: Differentially Expressed Circulating miRNAs in Cases vs Controls

miRNA	Fold Change	P-value	AUC (95% CI)
miR-126	2.8	<0.001	0.84 (0.78-0.90)
miR-199a	3.2	<0.001	0.82 (0.76-0.88)
miR-122	-2.1	<0.001	0.79 (0.73-0.85)
miR-141	2.5	<0.001	0.77 (0.71-0.83)
miR-145	-1.8	0.002	0.75 (0.69-0.81)

Table 3: Correlation between miRNA Expression and Clinical Parameters

miRNA	Disease Stage (r)	Infertility Duration (r)	CA-125 (r)
miR-126	0.68**	0.54**	0.62**
miR-199a	0.72**	0.58**	0.65**
miR-122	-0.45*	-0.38*	-0.42*
miR-141	0.56**	0.42*	0.48*
miR-145	-0.41*	-0.35*	-0.39*
*p<0.05, **p<0.01, r = Spearman's correlation coefficient			

DISCUSSION

Our study identified five significantly dysregulated circulating miRNAs in women with endometriosis-associated infertility. Most notably, miR-126 and miR-199a showed the highest diagnostic accuracy with AUC values of 0.84 and 0.82, respectively. These findings align with previous research by Wang et al. (2019), who reported similar upregulation of miR-199a in endometriosis patients. The combination of these miRNAs demonstrated enhanced diagnostic accuracy compared to single miRNA markers, suggesting their potential utility as a panel for non-invasive diagnosis. Our study's significant upregulation of miR-126 (2.8-fold) corresponds with findings from Khalaj et al. (2019), who demonstrated its role in angiogenesis and endometriotic lesion development. Our results extend these findings by establishing a strong correlation between miR-126 expression and disease stage ($r=0.68$, $p<0.01$), suggesting its potential as a marker for disease progression.

The strong correlation between miR-199a expression and disease stage ($r=0.72$, $p<0.01$) represents a novel finding. Previous studies by Maged et al. (2018) focused primarily on its diagnostic potential without exploring stage-specific associations. This correlation suggests that miR-199a might be a valuable tool for monitoring disease progression and treatment response. Interestingly, we observed significant negative correlations for miR-122 and miR-145 with disease severity and infertility duration. These findings support the work of Hull and Nisenblat (2013), who proposed that the downregulation of specific miRNAs might contribute to disease pathogenesis through the dysregulation of target genes involved in endometrial receptivity.

The correlation between miRNA expression and infertility duration provides new insights into the molecular mechanisms linking endometriosis and fertility problems. Particularly, miR-199a showed a strong correlation with infertility duration ($r=0.58$, $p<0.01$), supporting findings by Greene et al. (2016) regarding its role in endometrial function and implantation. MiR-141's upregulation (2.5-fold) and its correlation with CA-125 levels ($r=0.48$, $p<0.05$) suggest its potential role in the inflammatory processes associated with endometriosis. This aligns with research by Giudice (2010), who emphasized the importance of inflammatory markers in endometriosis pathogenesis.

The identification of this miRNA panel offers several advantages over current diagnostic methods. First, the non-invasive nature of blood sampling represents a significant improvement over laparoscopic diagnosis. Second, the strong correlations with disease stage and clinical parameters suggest potential utility in monitoring disease progression and treatment response. Our findings build upon previous work by Rogers et al. (2017), who highlighted the need for reliable biomarkers in endometriosis. The combination of differentially expressed miRNAs identified in our study achieved higher diagnostic accuracy than

single markers reported in earlier studies (Turchinovich et al., 2019).

Study Limitations

Several limitations should be considered when interpreting our results. The cross-sectional nature of the study prevents conclusions about causality. Additionally, the influence of hormonal variations across the menstrual cycle on miRNA expression requires further investigation through longitudinal studies. However, our study's strengths include its well-characterized patient population, standardized sample collection procedures, and comprehensive statistical analysis. Including correlation analyses with clinical parameters enhances the clinical relevance of our findings.

Future Research Directions

Further research is needed to validate these findings in larger, multicenter cohorts and to investigate the functional roles of these miRNAs in endometriosis pathogenesis. Longitudinal studies examining miRNA expression changes during treatment could provide valuable insights into their utility as prognostic markers.

CONCLUSION

This comprehensive analysis identifies a panel of five circulating miRNAs as potential non-invasive biomarkers for endometriosis-associated infertility. The strong correlations with clinical parameters and high diagnostic accuracy suggest their utility in disease monitoring and diagnosis. These findings provide a foundation for developing non-invasive diagnostic tools, though larger validation studies are warranted.

CONFLICTS OF INTEREST
None

ACKNOWLEDGMENT
None

AUTHORS CONTRIBUTION
All authors have equal contribution.

SOURCE OF FUNDING
None

DATA AVAILABILITY
None

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How to Cite:

Chauhan N. (2023). Investigation of circulating microRNAs as Potential Biomarkers for Endometriosis Associated Infertility *International Journal of Multidisciplinary Health Sciences and Research*, 1(1),16-22.